STUDIES TOWARDS THE COMPLETE SEQUENCE DETERMINATION OF PROTEINS BY MASS SPECTROMETRY; A RAPID PROCEDURE FOR THE SUCCESSFUL PERMETHYLATION OF HISTIDINE CONTAINING PEPTIDES

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1. Introduction

It is now widely recognised that mass spectrometric sequencing, involving the use of *N*-acetyl permethylated peptide derivatives, offers a viable alternative to classical approaches to the primary structure determination of proteins. The method does not as yet enjoy unanimous support, and critics may point to two areas of weakness. The first of these involves strategy. Comparison of the classical Dansyl–Edman procedure [1] with the mass spectrometric approach to the sequenching of *individual* peptides shows that, with a few exceptions (e.g. peptides with blocked N-termini), mass spectrometry offers no significant advantages in time-saving or sensitivity. Recently, however, we have described a strategy (mixture analysis) [2] which offers the distinct advantage of rapid sequence assignment, pre-empting the necessity for purification and isolation of individual peptides. The second, more fundamental weakness is associated with the derivatisation of the peptides, in particular the permethylation reaction. The best permethylation procedure to date has been the Hakamori reaction [3], first described for use on peptides by Vilkas and Lederer [4]. A modified procedure described by Thomas [5] is now commonly used, but this does not give satisfactory results with certain of the amino acids normally found in proteins. One of the troublesome amino acids is histidine, which is believed to form a quaternised salt on permethylation [6]. Using the Thomas procedure [5, 7] one can at best determine the sequence up to but not including the histidine, and more often no sequence information is obtained at all.

This report describes some experiments which have led to a successful derivatisation procedure for histidine-containing peptides, which has been tested by examination of several protein-derived peptides without prior knowledge of their sequences. In the following discussion, the advantages and possible disadvantages of this new procedure are evaluated.

2. Experimental

The methyl sulphinyl carbanion base used in all the experiments was prepared as described previously [5, 2]. No precautions were taken to exclude air during this preparation, and a honey coloured solution of strength 0.75–1.0 molar is normally obtained. The colour is due to decomposition products. The exact strength of the base has not been found to be critical, providing that an excess over peptide is used.

All mass spectra were recorded on an A.E.I.–G.E.C. MS902 instrument, operating at an accelerating voltage of 8 kV and an electron beam energy of 70 eV. The sample handling technique has been described previously [2].

3. Results

A number of experiments were carried out; the ones leading to the development of the proposed procedure are outlined below.

3.1. *The rate of methylation*

The recommended reaction time following addition of methyl iodide to the peptide/base mixture has

varied from 30 min [7] to 12 hr [4], but that recommended by Thomas [5], 1 hr, has generally been adopted.

1 mg of the peptide mixture below

Val–Gly–Leu–Ala–Pro–Val–Ala (I)
Ala–Asn–Asn–Lys (II)
Gln–Tyr–Tyr–Thr–Val–Phe–Asp–Arg (III)

was acetylated in the usual manner [6], and a 10-fold excess of base added. This was followed by the addition of 0.3 ml of CD_3I and the reaction mixture shaken for 60 sec. At this point, 0.3 ml of CH_3I was added, and the reaction allowed to continue for a further 59 min (total time 1 hr). The reaction products were extracted [2] and examined in the mass spectrometer.

The spectra showed that only CD_3 had been incorporated into the peptides, at least as far as the molecular ion region. This important result indicates that methylation at almost all the anionic sites created on the peptide can be regarded as instantaneous. Evidence of partial incorporation of CH_3 was present in the molecular ion region of peptide (II) (the ϵ-$NHCOCH_3$ of lysine is slow to methylate even under normal conditions), and also to a smaller degree in peptide (I). This indicates that the carboxylate anion of peptide (I) was not fully methylated during a 60 sec reaction time (see Discussion).

3.2. Following the incomplete methylation of the carboxyl group of peptide (I), several peptides containing acidic amino acids (Asp and Glu) were acetylated and permethylated using a 60 sec methylation time. The peptides were examined as unknowns, and completely satisfactory spectra were produced using 100 nmoles of material. The following sequences were unequivocably established.

Val–Gly–Lys–Asp–Leu–Thr–Leu
Leu–Asp–Leu–Phe
Glu–Pro–Val–Tyr
Val–Asp–Leu–Thr

3.3. A histidine-containing peptide was examined under normal reaction conditions [5]. No interpretable spectrum was produced.

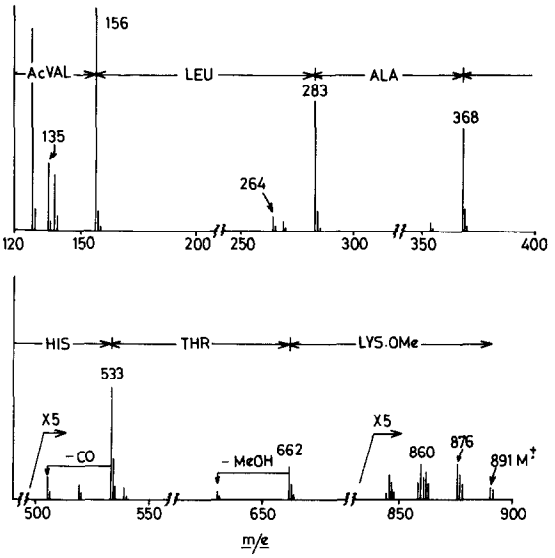


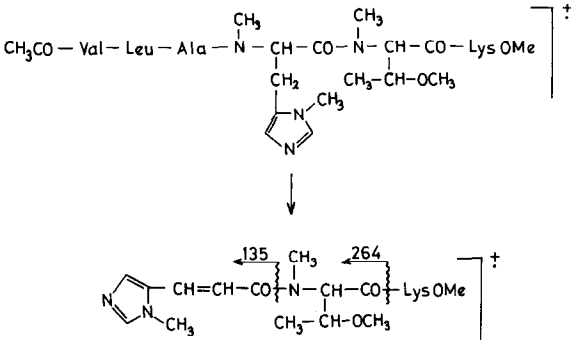
Fig. 1. Mass spectrum (above m/e 120) of a histidine-containing peptide showing the sequence Val–Leu–Ala–His–Thr–Lys. Source temperature 245°.

3.4. The same peptide (0.1 μmole) was derivatised as in 3.2, and a clear spectrum was produced, indicating the sequence

His–Ala–Asn–Ala–Gly–Ala–Tyr

This sequence has been confirmed.

3.5. Following the above experiments, several histidine-containing peptides were examined as unknowns under the new reaction conditions.



Scheme 1. The origin of m/e 135 and 264 in fig. 1, involving N–C cleavage and hydrogen rearrangement.

Fig. 1 shows the spectrum of one of these. The sequence is clear and unambiguous. The abundant ions at m/e 156, 283, 368, 533, 662 and 891 indicate the sequence

Val–Leu–Ala–His–Thr–Lys–OMe

Ions at m/e 877, 862 and 846 indicate the presence of some material unmethylated at the ϵ-position on lysine, but no problem arises because of this.

The relatively small ions at m/e 135 and 264 arise from the fragmentation shown in scheme 1, and can offer a useful confirmation of the sequence assignment.

Other histidine-containing peptides examined were shown to have the sequences below:

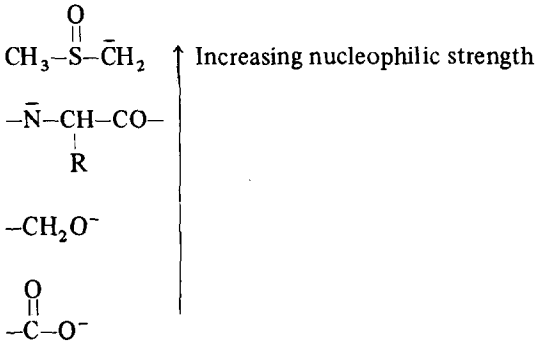
Ser –Ala–His–Lys–OMe
Asn–Ala–His–Leu–Gly–Val–Tyr–OMe
Ala –His–Ala–Glu–Lys–OMe

These peptides were available in insufficient quantity (0.1 μmole) to run duplicate experiments (3.3 and 3.4) but experience suggests that these sequences would not have been forthcoming had the peptides been derivatised in the normal manner.

4. Discussion

The results show that the optimal reaction time for peptide permethylation, using modifications of the Hakamori procedure, has been greatly overestimated, and that this has been a crucial factor in the failure to sequence histidine-containing peptides.

One can predict that carboxylate anion will be slow to methylate, as was seen in 3.1, since it is a weak nucleophile in comparison to other anionic centres present in the reaction medium (see below).



The experiments carried out show that the rate of quaternisation is appreciably slower than this, and that the choice of a suitable reaction time may result in a predominantly fully methylated but non-quaternised product.

The fact that preventing quaternisation in this manner may lead to non-quantitative esterification of carboxylic acid functions has not been a problem in the examples studied. In all cases, molecular ions (indicating a completely esterified C-terminal carboxyl group) were apparent at the 100 nmolar level. Should it be felt necessary, one could of course quantitatively esterify, following the permethylation procedure suggested.

To date, there have been two other suggestions for dealing with the histidine problem. One, due to Vliegenthart and Dorland [8] involves ring opening of the imidazole using diethyl pyrocarbonate under fairly vigorous conditions. This is followed by derivatisation in the normal manner. A second more recent suggestion [9] involves the Hakamori procedure, modified by accurately balancing the amount of base used with the amount of methyl iodide added to the reaction mixture. A normal reaction time is used. The authors claim that quaternisation is avoided under these conditions. The procedure necessitates the measurement of base strength, and the accurate measurement and careful handling of very small volumes of methyl iodide. In this laboratory, we have found this to be rather difficult and our results somewhat erratic.

The procedure suggested in this paper is rapid and simple, involving neither additional reaction steps nor more accurate measurements of reagents used, and is therefore convenient when dealing with large numbers of samples from protein hydrolysates.

Preliminary experiments suggest that the pyrimidyl ornithine derivative of arginine [10] is also successfully derivatised under these conditions.

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